Original Article

Intracellular mechanisms mediating tocotrienol-induced apoptosis in neoplastic mammary epithelial cells

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Tocotrienols and tocopherols represent the two subgroups that make up the vitamin E family of compounds. However, tocotrienols display significantly more potent apoptotic activity in neoplastic mammary epithelial cells than tocopherols. Studies were conducted to determine the intracellular mechanism(s) mediating tocotrienol-induced apoptosis in neoplastic +SA mouse mammary epithelial cells *in vitro*. An initial step in apoptosis is the activation of "initiator" caspases (caspase-8 or -9) that subsequently activate "effector" caspases (caspase-3, -6 and -7) and induce apoptosis. Treatment with cytotoxic doses of γ-tocotrienol (20 μM) resulted in a time-dependent increase in caspase-8 and caspase-3 activity. Combined treatment with specific caspase-8 or caspase-3 inhibitors completely blocked γ-tocotrienol-induced apoptosis and caspase-8 or caspase-3 activity, respectively. In contrast, γ-tocotrienol treatment had no effect on caspase-9 activation, and combined treatment with a specific caspase-9 inhibitor did not block γ-tocotrienol-induced apoptosis in +SA cells. Since caspase-8 activation is associated with the activation of death receptors, such as Fas, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL) receptors, studies were conducted to determine the exact death receptor(s) and ligand(s) involved in mediating tocotrienol-induced caspase-8 activation and apoptosis. Treatment with Fas-ligand (FasL), Fas-activating antibody, or TRAIL failed to induce cell death in +SA neoplastic mammary epithelial cells, suggesting that these cells are resistant to death receptor-induced apoptosis. Moreover, treatment with cytotoxic doses of γ-tocotrienol did not alter the intracellular levels of Fas, FasL, or Fas-associated death domain (FADD) in these cells. Western blot analysis also showed that γ-tocotrienol did not induce FasL or FADD translocation from the cytosolic to membrane fraction in these cells. Finally, treatment with Fas-blocking antibody did not reverse the tocotrienol-induced apoptosis in + SA cells. These data demonstrate that tocotrienol-induced caspase-8 activation and apoptosis is not mediated through death receptor activation in malignant +SA mammary epithelial cells. Resistance to death receptor-induced apoptosis has been shown to be associated with increased expression of apoptosis-inhibitory proteins, such as FLICE-inhibitory protein (FLIP), and enhanced signalling of the phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase (PDK)/Akt mitogenic pathway. Additional studies showed that treatment with cytotoxic doses of γ-tocotrienol decreased total, membrane, and cytosolic levels of FLIP, and reduced phosphorylated PDK-1 (active) and phosphorylated-Akt (active) levels in these cells. In summary, these findings demonstrate that tocotrienol-induced caspase-8 activation and apoptosis in malignant +SA mammary epithelial cells is not mediated through the activation of death receptors, but appears to result from the suppression of the PI3K/PDK/Akt mitogenic signalling pathway, and subsequent reduction in intracellular FLIP expression.

Key Words: vitamin E, tocotrienols, breast cancer, apoptosis, caspase, PDK, Akt, FLIP

Introduction

Tocopherols and tocotrienols represent the two subgroups in the vitamin E family of compounds.[1,2] Both subgroups have the same basic chemical structure characterized by a long phytyl tail attached to a chromane ring. However, tocopherols have a saturated, whereas tocotrienols have an unsaturated phytyl tail. Although both are potent antioxidants, only tocotrienols display potent antiproliferative and apoptotic activity against breast cancer cells, and these effects are observed using treatment doses that have little or no effects on normal mammary epithelial cell growth or viability.[1-4] Recently, studies have demonstrated that tocotrienol-induced cell death results from the initiation of apoptosis.[1-4] Apoptosis or programmed cell death is an important mechanism by which cancer cells can be eliminated from the body, and is characterized by organized and systematic cellular destruction brought about by the activation of specific enzymes called caspases.[5, 6]

Caspase activation can result from two basic mechanisms. The first mechanism involves receptor-mediated caspase activation. Activation of "death receptors", such as Fas, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL) receptors by their specific ligands results in receptor trimerization, recruitment of adapter proteins such as Fas-associated death domain (FADD), and

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inactive initiator caspases (procaspase-2 or -8), to form what is called a death inducing signalling complex (DISC).[7] The initiator caspase within the DISC is then activated and released. Activated initiator caspases then activate effector caspases, such as capase-3, which then mediate the various cytoplasmic and nuclear events associated with apoptosis.[7] Studies have shown that stimulation of the phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase (PDK)/Akt mitogenic signalling pathway results in the increased intracellular expression of FLICE-inhibitory protein (FLIP), a cytoplasmic protein that prevents procaspase-8 activation.[8-10] Treatments that inhibit PI3K/PDK/Akt mitogenic signalling can cause a reduction in intracellular FLIP levels, and a corresponding increase in caspase-8 activation and apoptosis.[8-10] The second mechanism involves mitochondrial stress-induced caspase activation. Various stressful stimuli (chemicals or radiation) can cause perturbations in the mitochondrial membrane that results in the release of pro-apoptotic molecules, such as cytochrome c, into the cytoplasm.[11,12] Cytochrome c will then interact with cytoplasmic proteins to induce the activation of the initiator caspases, such as caspase-9, which subsequently activates down-stream effector caspases.[11,12] It has also been shown that cross-talk between the receptor-mediated and mitochondrial stress pathways can occur in some apoptotic models. Activation of upstream initiator or down-stream effector caspases has been shown to directly lead to the release of cytochrome c from the mitochondria, and subsequent caspase-9 activation in some cells.[13-16] Studies were conducted to determine whether death receptor and/or mitochondrial stress-mediated signalling pathways are involved in tocotrienol-induced apoptosis.

Material and Methods

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. The highly malignant mouse +SA mammary epithelial cell line was used in these studies as previously described in detail.[3,4] Cells were maintained in serum-free control media consisting of DMEM/F12 containing 5mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF), and 10 μg/ml insulin. Cells were plated at a density of 1 X 10^5 cells/well in 24-well culture plates (viability studies) or 1 X 10^6 cells/100 mm culture plates (DNA fragmentation, Western blot, and caspase enzymatic activity studies). In order to dissolve the highly lipophilic vitamin E compounds in aqueous culture media, these compounds were conjugated to BSA as previously described.[1,3,17-19] This solution of vitamin E conjugated to BSA was then used to prepare various concentrations of (0-400μM) α-tocopherol or (0-20 μM) γ-tocotrienol supplemented treatment media such that all control and treatment media had a final concentration of 5 mg/ml BSA. Ethanol was added to all treatment media such that the final ethanol concentration was the same in all treatment groups within a given experiment and was always less than 0.1%. In combination studies, caspase inhibitors were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Stock solutions (1 mM) of specific caspase-8 inhibitor II, benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (z-IETD-fmk); and caspase-3 inhibitor V, benzyloxycarbonyl - Asp (OMe) - Gln - Met - Asp(OMe)-fluoromethyl ketone (z-DQMD-fmk) were prepared in dimethyl sulfoxide (DMSO), and added to the culture media so that the final concentration of each inhibitor was 1μM. Appropriate amounts of DMSO were also added to control media so that the final concentration of DMSO was the same in all treatment groups. Cells were divided into different treatment groups and fed serum-free control or treatment media every other day and maintained in a humidified incubator at 37°C in an environment of 95% air and 5% CO_2. Viable cell number was determined in the different treatment groups by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously.[1,3,17-19] Differences among the various treatment groups were determined by analysis of variance, followed by Duncan's multiple range test. A difference of $P < 0.05$ was considered to be significant, as compared with controls or as defined in the Figure legends.

Treatment-induced caspase activation was determined using caspase-8 and caspase-3 colorimetric assay kits purchased from Clontech Laboratories (Palo Alto, CA), and the caspase-9 colorimetric assay kit purchased from R&D Systems (Minneapolis, MN). Malignant +SA mammary epithelial cells were divided into different treatment groups and incubated with control or treatment media for 0-24 hr. After treatment exposure, cells were isolated from plates with trypsin, and then lysed on ice for 10 min using the cell lysis buffer provided in the caspase assay kits. The lysate was sonicated for 15 sec on ice and centrifuged for 1 min at 14,000 X g at 4°C. Whole cell lysates were then separated from cellular debris and stored at -80°C until assayed for caspase activity. Protein concentrations in whole cell lysates were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and 200μg of protein was used to measure caspase activity. The colorimetric enzymatic activity was read as optical density units at 405 nm on a microplate reader, according to the instructions provided in each kit.

Western Blot analysis was used to determine treatment effects on the intracellular levels of Fas, Fas-L, TRAIL, active forms of intracellular kinases (phosphorylated-PDK1 and phosphorylated-Akt), and intracellular proteins (FADD and FLIP) associated with stimulating or inhibiting apoptotic signalling. Malignant +SA mammary epithelial cells in the various treatment groups were isolated from culture plates with trypsin, whole cell lysates were prepared, and protein concentration of each lysate was determined as previously described.[4,19,20] Samples from each treatment group (20 μg/lane) were loaded on 10-15% polyacrylamide minigels, electrophoresed, and proteins were transblotted (30 Volts for 12 hr) to polyvinylidene difluoride (PVDF) membranes (Dupont, Boston, MA) according to the method of Towbin *et al*.[21] To identify various intracellular proteins, membranes were blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20 pH 7.4, and incubated for 2 hr with either Fas polyclonal antibody (1:4000, Santa Cruz Biotechnology, Santa Cruz, CA), Fas-ligand (FasL) polyclonal antibody (1:4000, Santa

Cruz Biotechnology, Santa Cruz), FADD monoclonal antibody (1:5000, Upstate, Inc., Lake Placid, NY), phosphorylated-PDK-1 polyclonal antibody (1:5000, Cell Signaling Technology, Beverly, MA), phosphorylated-Akt polyclonal antibody (1:5000, Cell Signalling Technology, Beverly, MA) or FLIP polyclonal antibody (1:4000, Upstate, Inc., Lake Placid, NY). Membranes were then rinsed five times with blocking buffer, and incubated for 1 hr with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5000) or goat anti-rabbit (1:5000) secondary antibody (Pierce, Rockford, IL). Blots were then rinsed five times with blocking buffer and protein bands were visualized with the SuperSignal enhanced chemiluminescence kit according to the manufacturer's instructions (Pierce, Rockford, IL). Blots from each treatment group were exposed on the same piece of film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with Epson Perfection 1640SU photo scanner (Epson, Long Beach, CA) and analyzed with QuantityOne software (Bio-Rad, Hercules, CA).

Results

The effects of 24 hr exposure to control, 400 μM α-tocopherol or 20 μM γ-tocotrienol treatment media on viable +SA cell number are shown in Figure 1. Cells in all treatment groups were plated at a concentration of 1 X 10^5 cells/well in 24-well culture plates and maintained on control media for 3 days in culture. Afterwards, control media was removed and replaced with their specific treatment media. The next day, cell viability in each treatment group was determined using the MTT colorimetric assay. Treatment for 24 hr with 400 μM α-tocopherol had no effect on malignant + SA mammary epithelial cell viability as compared to controls (Fig. 1). In contrast, treatment with 20 μM γ-tocotrienol resulted in over a 70% reduction in +SA cell viability as compared to controls (Fig. 1). Additional studies confirmed previous investigations[1,3,4] that showed γ-tocotrienol-induced cell death results from apoptosis, as indicated by DNA fragmentation and positive TUNEL assay staining (data not shown).

Figure 2 shows the effects of γ-tocotrienol treatment on caspase activation in malignant +SA mammary epithelial cells. Cells were grown in culture for 3 days in control media and then divided into different treatment groups and treated for 0-24 hr with either control or 20 μM γ-tocotrienol treatment media. Treatment with γ-tocotrienol induced a large increase in caspase-8 activity that was detected within 4 hr and continued for at least 24 hr after treatment exposure as compared to untreated controls (Fig. 2A).

Similar treatment with γ-tocotrienol also induced a corresponding increase in caspase-3 activity (Fig. 2C), but had no effect on caspase-9 activity at any time during the 24 hr treatment period (Fig. 2B) as compared to untreated controls. The effects of selective caspase inhibitors on γ-tocotrienol induced caspase activation in malignant +SA mammary epithelial cells are shown in Figure 3. Cells were grown in culture for 3 days in control media and then divided into different treatment groups and treated for- 24 hr with -either control, 1 μM z-IETD-fmk, 20 μM

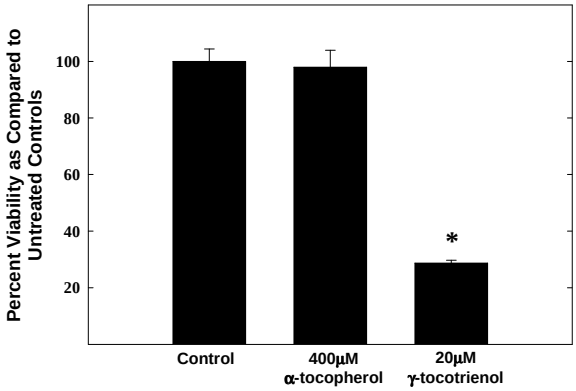

Figure 1. The percent of viable +SA cells following 24 hr treatment exposure to 400 μM α-tocopherol or 20 μM γ-tocotrienol, as compared to untreated controls. Cells (1 X 10^5 cells/well) in each treatment group (6 wells/group) were grown in serum-free control media for 3 days prior to exposure to their respective treatments. Vertical bars indicate the mean percent of viable cells ± SE. *P<0.05 as compared to untreated controls.

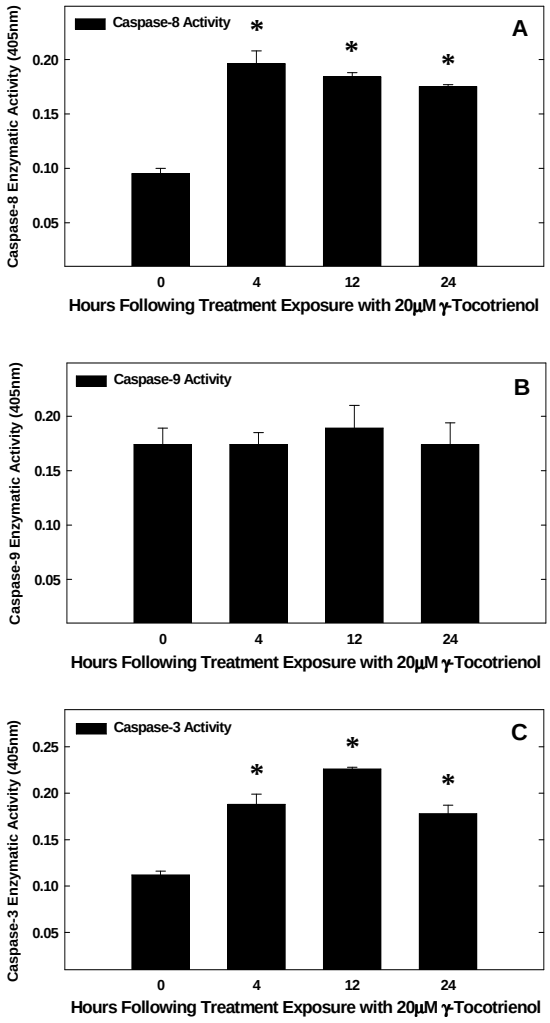

Figure 2. Caspase-8 (A), caspase-9 (B), and caspase-3 (C) activity in malignant +SA mammary epithelial cells following 0-24 hr exposure to 20 μM γ-tocotrienol. Caspase activity is expressed as optical density units at 405 nm. Vertical bars represent the means ± SD for duplicates in each treatment groups. *P<0.05 as compared to untreated controls.

γ-tocotrienol, or the combination of 1 μM z-IETD-fmk and 20 μM γ-tocotrienol treatment media (Fig. 3A). Cells treated with 1 μM z-IETD-fmk (I-8), a specific caspase-8 inhibitor, showed no change in the relative level of caspase-8 activity as compared to untreated controls (Fig.3A). In contrast, treatment with 20 μM γ-tocotrienol (γT3) resulted in a significant elevation in caspase-8 activity 24 hr following treatment exposure (Fig. 3A). Combined treatment with 1 μM z-IETD-fmk (I-8) and 20 μM γ-tocotrienol (γ T3) blocked tocotrienol-induced caspase-8 activation (Fig. 3A). In similar studies, malignant +SA mammary epithelial cells were exposed for 24 hr to control, 1 μM z-DQMD-fmk, 20 μM γ-tocotrienol, or the combination of 1 μM z-DQMD-fmk and 20 μM γ-tocotrienol treatment media. Treatment with 1 μM z-DQMD-fmk (I-3), a specific inhibitor of caspase-3, had no significant effect, while treatment with 20 μM γ-tocotrienol (γT3) induced a significant increase in caspase-3 activity 24 hr following treatment exposure as compared to untreated controls (Fig. 3B). Combined treatment with 20 μM γ-tocotrienol (γT3) and 1 μM z-DQMD-fmk (I-3) blocked tocotrienol-induced caspase-3 activation (Fig. 3B).

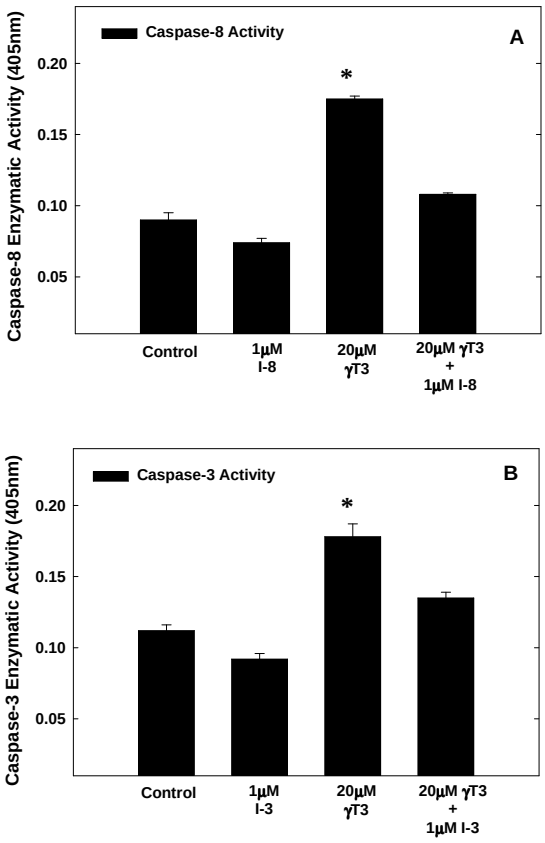

Figure 3. Caspase-8 (A) activity in malignant +SA mammary epithelial cells following a 24 hr exposure to control, 1 μM z-IETD-fmk (I-8, a specific caspase-8 inhibitor), 20μM γ-tocotrienol (γT3), or the combination of 1 μM z-IETD-fmk (I-8) and 20 μM γ-tocotrienol (γT3) treatment media. Caspase-3 (B) activity in +SA cells following a 24 hr exposure to control, 1 μM z-DQMD-fmk (I-3, a specific caspase-3 inhibitor), 20 μM γ-tocotrienol (γT3), or the combination of 1 μM z-DQMD-fmk (I-3) and 20 μM γ–tocotrienol (γ T3) treatment media. Caspase activity is expressed as optical density units at 405 nm. Vertical bars represent the mean ± SD of duplicates in each treatment group. *P<0.05 as compared to untreated controls.

Since caspase-8 processing and activation is associated with "death receptor" mediated apoptotic signalling, studies were conducted to determine which death receptor/ligand signalling mechanism was involved in tocotrienol-induced apoptosis. Cells were grown in culture for 3 days in control media and then divided into different treatment groups and treated for 24 hr with either control, 20 μM γ–tocotrienol, 1 μg/ml Fas-activating antibody (Fas-Ab), 100 ng/ml FasL or 100 ng/ml tumor TRAIL treatment media. Treatment with γ–tocotrienol significantly reduced the number of viable +SA mammary epithelial cells as compared to control, 24 hr after treatment exposure (Fig. 4). In contrast, treatment with Fas-Ab or FasL, agents that are known to activate Fas, had no effect on +SA cell viability as compared to untreated controls. In other studies, treatment with Fas-blocking antibody did not block γ-tocotrienol-induced apoptosis in these cells (data not shown). Treatment with TRAIL was also shown to have no effect on +SA cell viability as compared to untreated controls (Fig. 4). Since death receptor ligands (FasL and TRAIL) and activating antibody (Fas-Ab) were not found to induce apoptosis, these finding indicate that tocotrienol-induced caspase-8 activation and apoptosis occurs independently of death receptor activation in malignant +SA mammary epithelial cells.

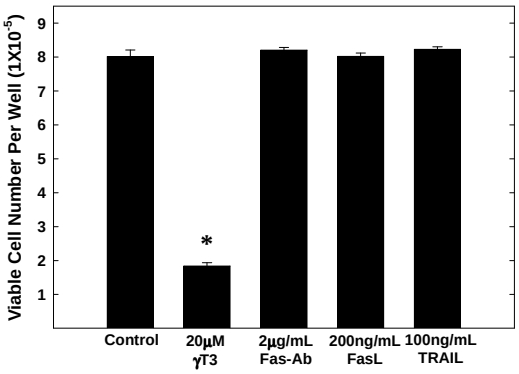

Figure 4. Viable +SA mammary epithelial cell number following a 24 hr exposure to control, 20 μM γ-tocotrienol (γ T3), 1μg/ml Fas-activating antibody (Fas-Ab), 100 ng/ml Fas ligand (FasL) or 100 ng/ml TRAIL treatment media. Cells (1 X 10^5 cells/well) in each treatment group (6 wells/group) were grown in serum-free control media for 3 days prior to exposure to their respective treatments. Vertical bars indicate the mean percent of viable cells ± SE. *P<0.05 as compared to untreated controls.

The effects of control and 20 μM γ-tocotrienol (γT3) on the relative cellular levels of Fas, FasL, FADD, phosphorylated-PDK1 (active), phosphorylated-Akt (active) and FLIP are shown in Figure 5. Cells in the different treatment groups were grown in culture for 3 days in serum-free control media. Afterwards, media was removed and replaced with their respective culture media. Following an 8 hr treatment period, cells in all treatment groups were isolated with trypsin, and whole cell lysates were prepared for Western blot analysis. Treatment with γ-–tocotrienol had no effect on the relative cellular levels of Fas or FasL (Fig. 5). Likewise, γ-tocotrienol treatment

had no effect on the relative intracellular levels of FADD, the adapter protein required for Fas activation of caspase-8 (Fig. 5). Western blot analysis also showed that γ-tocotrienol did not induce translocation of Fas-ligand or FADD from the cytosol to the cell membrane (data not shown). However, γ-tocotrienol treatment was found to induce a relatively large decrease in phosphorylated-PDK1 (active), phosphorylated-Akt (active), and FLIP levels as compared to untreated controls (Fig. 5).

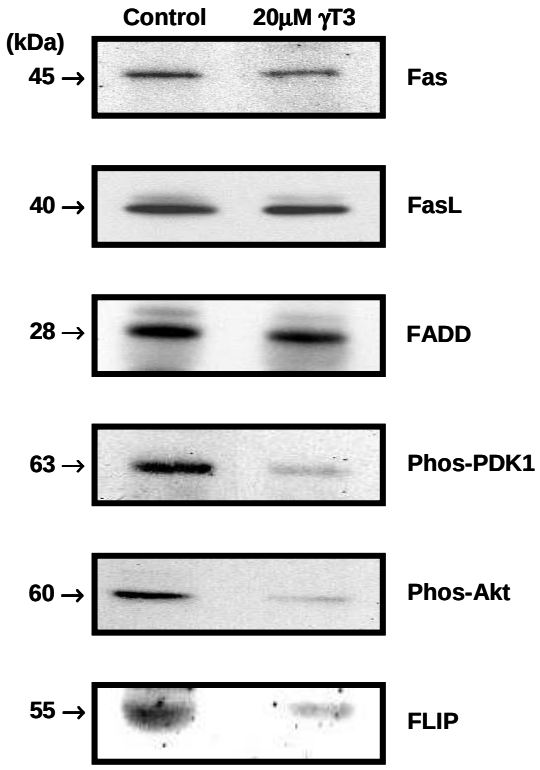

Figure 5. Effects of 0-20 μM γ-tocotrienol (γT3) on the relative intracellular levels of Fas, Fas ligand (FasL), FADD, phosphorylated-PDK1 (phos-PDK1, active), phosphorylated-Akt (phos-Akt, active) and FLIP in whole cell lysates following an 8 hr treatment exposure. +SA cells were grown in culture for 3 days in serum-free control media prior to treatment exposure. Whole cell lysates (20 μg/lane) from the different treatment groups were fractionated by SDS-PAGE and then protein bands were visualized by Western blot analysis.

Discussion

These results demonstrate that tocotrienol-induced apoptosis in highly malignant +SA mammary epithelial cells is mediated by specific intracellular signalling mechanisms that lead to sequential activation of caspase-8 and caspase-3. In contrast, treatment with very high doses of α-tocopherol had no effect on breast cancer cell viability. Furthermore, combined treatment with specific inhibitors of caspase-8 or caspase-3, were found to block γ-tocotrienol- induced apoptosis. Tocotrienol treatment had no effect on caspase-9 activity, and combined treatment with a specific caspase-9 inhibitor did not block tocotrienol-induced apoptosis in malignant +SA mammary epithelial cells. Since caspase-8 processing and activation is associated with death receptor apoptotic signalling[7] these findings suggested that tocotrienol-induced caspase activation might be mediated through Fas, TNF and/or TRAIL receptor apoptotic signalling mechanisms. However, treatment with antibodies or ligands that activate Fas or TRAIL receptors did not induce apoptosis in these cells. These findings indicate that the highly malignant +SA mammary epithelial cells are resistant to death receptor-induced apoptosis. However, additional studies showed that tocotrienol-induced caspase-8 activation is associated with tocotrienol-dependent inhibition of PI3K/PDK/Akt mitogenic signalling, and corresponding reduction in intracellular FLIP expression, an intracellular protein associated with preventing caspase-8 activation. These findings confirm and extend previous reports that showed tocotrienol-induced apoptosis is mediated through caspase-8 activation and independent of caspase-9 activation.[4] Since caspase-8 activation is associated with death receptor apoptotic signalling, whereas caspase-9 activation is associated with mitochondrial stress-induced apoptotic signalling[5-7], it was hypothesized that tocotrienol-induced apoptosis resulted from death receptor-dependent signalling mechanisms.[4] However, this report had not determined the exact death receptor/ligand signalling mechanisms involved in tocotrienol-induced apoptosis.[4] It is known that Fas activation plays an important role in the induction and regulation of apoptosis in the mammary gland during development, involution, and tumorigenesis.[22-26] Fas activation induces translocation of inactive procaspase-8 from the cytoplasm to the cell surface death receptor, where it is then cleaved and processed to active caspase-8.[5,6,27,28] Fas activation can be initiated by Fas-ligand (FasL), or activating anti-Fas antibody that binds to Fas and stimulate apoptotic signalling. Other investigators have shown that α-tocopherol succinate, a synthetic derivative of α-tocopherol, stimulates Fas signalling and apoptosis in other cell lines.[26] However, results in the present study demonstrate that the Fas receptor is non-functional in malignant +SA mammary epithelial cells. Treatment with FasL or Fas activating antibody did not induce apoptosis or decrease viable cell number. Tocotrienol-induced apoptosis was also not associated with alterations in intracellular Fas, FasL or FADD levels in these cells. Treatment with TRAIL had previously been shown to induce caspase-8 activation through death receptor mediated mechanisms in several experimental models.[29] However, treatment with high doses of TRAIL did not induce apoptosis or decrease cell viability in +SA cells. These findings clearly demonstrate that tocotrienol-induced caspase-8 activation and apoptosis is not mediated through Fas or TRAIL receptor apoptotic signalling mechanisms in the highly malignant +SA mammary epithelial cells.

Apoptosis in normal and neoplastic mammary epithelial cells can be initiated through multiple signalling pathways.[30-33] It is well established that mitogen-starved mammary epithelial cells will undergo apoptosis approximately 15 hr after mitogens are removed from the culture medium.[32] Studies have shown that certain mitogens induce the synthesis and/or expression of anti-apoptotic proteins that prevent apoptosis and increase cell survival.[32] Several mitogen-dependent anti-apoptotic signalling pathways have been identified, including the PI3K/PDK/Akt mitogenic signalling pathway.[8-10] Activation of receptor tyrosine kinases, such as the epidermal

growth factor (EGF) receptor, stimulates activation of PI3K.[8-10] PI3K then phosphorylates and activated PDKs (PDK1 and PDK2). Activation of PDK1 has been shown to phosphorylate and activate Akt[8-10] and activated Akt increases intracellular FLIP expression, an anti-apoptotic cytoplasmic protein that prevents the conversion of inactive procaspase-8 to active caspase-8.[8-10] A schematic representation of this pathway is shown in Figure 6.

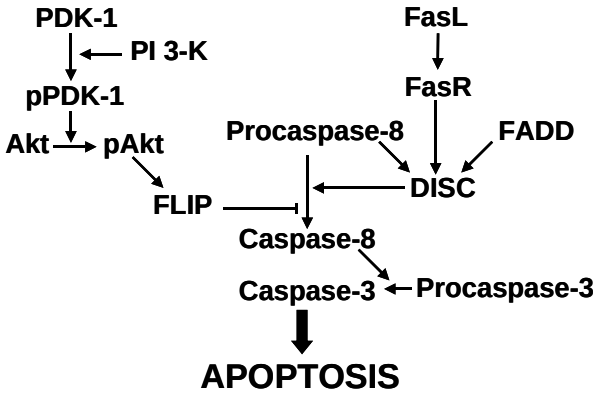

Figure 6. A schematic representation of caspase-8 and caspase-3 activation by either Fas death receptor apoptotic signalling or by the inhibition of the PI3K/PDK/Akt mitogenic pathway and subsequent reduced in FLIP expression.

Treatment with 20 μM γ-tocotrienol, a dose that induced a 70% reduction in +SA cell viability within 24 hr after treatment exposure, caused a large relative decrease in active PDK1 and Akt, and a corresponding reduction in intracellular FLIP expression in malignant +SA mammary epithelial cells. These results strongly suggest that tocotrienol-induced caspase-8 activation and apoptosis is mediated through the inhibition of the EGF-dependent PI3K/PDK/Akt mitogenic signalling, and subsequent reduction in mitogen-dependent expression of anti-apoptotic proteins. This hypothesis would also explain tocotrienol-induced caspase-8 activation in the absence of functional Fas or TNF receptors in malignant +SA mammary epithelial cells. Previous studies showed that tocotrienol-induced suppression of EGF-dependent cell proliferation did not result from a reduction in EGF-receptor levels or tyrosine kinase activity, suggesting that the antiproliferative and apoptotic effects of tocotrienols occurs downstream of the EGF-receptor.[18,19] Although γ-tocotrienol was clearly found to inhibit PDK1 activation in these cells, further studies are required to determine if α -tocotrienol directly inhibits PDK1 activation or if it is acting upstream to inhibit PI3K activity and subsequent PDK1 activation.

In summary, activation of initiator caspase-8 and effector caspase-3 are required for tocotrienol-induced apoptosis in malignant +SA mouse mammary epithelial cells. However, tocotrienol-induced caspase-8 activation occurs independently of death receptor activation and apoptotic signalling. Tocotrienol-induced caspase-8 activation and apoptosis appears to result from the inhibition of the PI3K/PDK/Akt mitogenic signalling, and subsequent reduction in intracellular FLIP levels. Tocotrienol-induced reductions in intracellular FLIP levels appear to lead to the dysinhibition of procaspase-8 activation, increased intracellular caspase-8 activity, and ultimately the initiation of apoptosis in these cells. Since the apoptotic effects of tocotrienols are observed using treatment doses that have been shown to have little or no effect on normal mammary epithelial cells growth or viability,[1,2,18] these findings suggest that dietary supplementation of tocotrienols may act to reduce the risk of breast cancer in women. However, additional studies are required to further determine the exact intracellular mechanism(s) mediating the antiproliferative and apoptotic effects of tocotrienols in order to provide essential information necessary for clearly understanding the potential health benefits of these compounds in the prevention and treatment of breast cancer.

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References

1. McIntyre BS, Briski KP, Tirmenstein MA, Fariss MW, Gapor A, Sylvester PW. Antiproliferative and apoptotic effects of tocopherols and tocotrienols on normal mouse mammary epithelial cells. Lipids 2000; 35:171.
2. Sylvester PW, Theriault A. Role of tocotrienols in the prevention of cardiovascular disease and breast cancer. Curr Top Nutraceutical Res 2003; 1:121.
3. McIntyre BS, Briski KP, Gapor, A, Sylvester PW. Antiproliferative and apoptotic effects of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. Proc. Soc S. Exp Biol Med E.B.M. 2000; 224: 292.
4. Shah S, Gapor A, Sylvester PW. Role of caspase-8 activation in mediating vitamin E-induced apoptosis in murine mammary cancer cells. Nutr Cancer 2003; 45: 236.
5. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 1999; 68: 383.
6. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999; 6: 1067.
7. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. Eur J Biochem 1998; 254: 439.
8. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J Biol Chem 2001; 276: 6893.
9. Suhara T, Mano T, Oliverira BE, Walsh K. Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). Circ Res 2001; 89: 13.
10. Varadhachary AS, Edidin M, Hanlon AM, Peter ME, Krammer PH, Salgame P. Phosphatidylinositol 3'-kinase blocks CD95 aggregation and caspase-8 cleavage at the death-inducing signaling complex by modulating lateral diffusion of CD95. J Immunol 2001; 166: 6564.
11. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309.
12. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997: 91: 479.

13. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 1999; 144: 891.
14. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998; 94: 491.
15. Chen Q, Gong B, Almasan A. Distinct stages of cytochrome c release from mitochondria: evidence for a feedback amplification loop linking caspase activation to mitochondrial dysfunction in genotoxic stress induced apoptosis. Cell Death Differ 2000; 7: 227.
16. Kirsch DG, Doseff A, Chau BN, Lim DS, de Souza-Pinto NC, Hansford R, Kastan MB, Lazebnik YA, Hardwick JM. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem 1999; 274: 21155.
17. Sylvester PW, Birkenfeld HP, Hosick HL, Briski KP. Fatty acid modulation of epidermal growth factor-induced mouse mammary epithelial cell proliferation in vitro. Exp Cell Res 1994; 214: 145.
18. Sylvester PW, McIntyre BS, Gapor A, Briski KP. Vitamin E inhibition of normal mammary epithelial cell growth is associated with a reduction in protein kinase C(alpha) activation. Cell Prolif 2001; 34: 347.
19. Sylvester PW, Nachnani A, Shah S, Briski KP. Role of GTP-binding proteins in reversing the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells. Asia Pacific J Clin Nutr 2002; 11: S452.
20. McIntyre BS, Birkenfeld HP, Sylvester PW. Relationship between epidermal growth factor receptor levels, autophosphorylation and mitogenic-responsiveness in normal mouse mammary epithelial cells in vitro. Cell Prolif 1995; 28: 45.
21. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76: 4350.
22. Keane MM, Ettenberg SA, Lowrey GA, Russell EK, Lipkowitz S. Fas expression and function in normal and malignant breast cell lines. Cancer Res 1996; 56: 4791.
23. Shen K, Novak RF. Fas-signaling and effects on receptor tyrosine kinase signal transduction in human breast epithelial cells. Biochem Biophys Res Commun 1997; 230: 89.
24. Kumar R, Vadlamudi RK, Adam L. Apoptosis in mammary gland and cancer. Endocr Relat Cancer 2000; 7: 257.
25. Kline K, Yu W, Sanders BG. Vitamin E: mechanisms of action as tumor cell growth inhibitors. J Nutr 2001; 131: 161S.
26. Yu W, Israel K, Liao QY, Aldaz CM, Sanders BG, Kline K. Vitamin E succinate (VES) induces Fas sensitivity in human breast cancer cells: role for Mr 43,000 Fas in VES-triggered apoptosis. Cancer Res 1999; 59: 953.
27. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J Biol Chem 1999; 274: 5053.
28. Bratton SB, MacFarlane M, Cain K, Cohen GM. Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. Exp Cell Res 2000; 256: 27.
29. Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 2000; 256: 58.
30. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990; 348: 334. +
31. Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes [comment]. Cell 1994; 79: 189.
32. Nass SJ, Li M, Amundadottir LT, Furth PA, Dickson RB. Role for Bcl-xL in the regulation of apoptosis by EGF and TGF beta 1 in c-myc overexpressing mammary epithelial cells. Biochem Biophys Res Commun 1996; 227: 248.
33. Heermeier K, Benedict M, Li M, Furth P, Nunez G, Hennighausen L. Bax and Bcl-xs are induced at the onset of apoptosis in involuting mammary epithelial cells. Mech Dev 1996; 56: 197.

Intracellular mechanisms mediating tocotrienol-induced apoptosis in neoplastic mammary epithelial cells
生育三烯酸调节新生乳房上皮瘤细胞程序性死亡的胞内机理

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生育三烯酚类和生育酚这两类物质都属于维生素 E 族。但是，在促使乳房上皮瘤细胞凋亡方面，生育三烯酚类显然要比生育酚效果好。本研究通过+SA 小鼠新生乳房上皮瘤细胞的体外培养，对生育三烯酚类的调节机制进行了探讨。编程性细胞死亡的第一步是激活启示胱门蛋白酶（8－胱门蛋白酶或 9－胱门蛋白酶），然后胱门蛋白酶再激活效应胱门蛋白酶（3－，6－或 7－胱门蛋白酶），这样就引起了编程性细胞死亡。用与细胞毒素当量的剂量（20 μM）的γ-生育三烯酸处理+SA 小鼠新生乳房上皮瘤细胞，结果 8－半胱天冬酶和 3－细胞凋亡蛋白酶的活性随着处理的时间增长而增强。如果同时用专性 8－半胱天冬酶或 3－细胞凋亡蛋白酶抑制剂来处理小鼠细胞，则能完全抑制γ-生育三烯酸引起的编程性细胞死亡以及 8－半胱天冬酶或 3－细胞凋亡蛋白酶的活性。相反，用γ-生育三烯酸处理后，并没有抑制 9－半胱天冬酶的活性，并且与专性 9－半胱天冬酶抑制剂同时处理细胞时，不会抑制γ-生育三烯酸引起的编程性细胞死亡。由于 8－半胱天冬酶的活性与编程性细胞死亡受体（比如 Fas,TNF 或 TRAIL 受体）的活性相关，许多研究就对参予调节生育三烯酸引起的 8－半胱天冬酶的活性和编程性细胞死亡的死亡受体和配体进行研究。用 Fas 配体，Fas 的激活抗体或者 TRAIL 处理+SA 小鼠新生乳房上皮瘤细胞后，并不能引起编程性细胞死亡，结果表明这些细胞能够耐死亡受体引起的编程性细胞死亡。并且用细胞毒素剂量的γ-生育三烯酸处理小鼠细胞后，并没改变这些细胞内的 Fas,FasL 和 FADD 的含量。Western blot 结果表明γ-生育三烯酸并不影响 FasL 和 FADD 在胞质与细胞膜之间的传输。最终，Fas 的抑制抗体并不能阻止生育三烯酸在+ SA 小鼠细胞中引起的编程性细胞死亡。这些结论表明生育三烯酸并不是通过调节编程性细胞死亡受体的活性来引起+SA 小鼠新生乳房上皮瘤细胞的编程性细胞死亡以及提高 8－半胱天冬酶的活性的。同时我们还得出对由死亡受体引起的程序性细胞死亡的抵抗性与编程性细胞死亡抑制性蛋白（如 FLIP）的高表达量和提高 PI3K/ PDK/Akt 信号输出相关的结论。除斥之外，我们还发现用细胞毒素当量的生育三烯酸处理小鼠细胞后，FLIP（包括总的 FLIP，细胞膜 FLIP 和胞质 FLIP）的含量以及 PDK-1 和 Akt 的磷酸化酶的活性会降低。总之，本研究得出了以下结论： +SA 小鼠新生乳房上皮瘤细胞的程序性死亡以及 8－半胱天冬酶的活性的提高并不是由生育三烯酸通过调节编程性细胞死亡受体的活性来引起，而是可能通过对 PI3K/PDK/Akt 丝裂信号传递途径的抑制，从而降低胞内 FLIP 的表达量所致。

关键词：维生素 E，生育三烯酚类，乳腺癌，编程性细胞死亡，半胱天冬酶，PDK, Akt, FLIP